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14. ABSTRACT

Genomic alterations of 18q have been observed in prostate cancer. This research focuses on analyzing the role of increased gene copy number at 18q22.1 in prostate cancer. We believe the key genes in this region are type II cadherins. We are studying the role of overexpression of cadherin 7 (*CDH7*), on the tumorigenic and invasive potential of prostate cancer cells. The increased copy number of *CDH7* is specific to prostate cancer and results in increased levels of cadherin-7 mRNA in prostate tumors. We created monoclonal antibodies against full-length bacterially-expressed protein and have tested these antibodies for their use in immunohistochemistry experiments with paraffin-embedded prostate tissue. We now have an adequate anti-CDH7 antibody with which we can survey a prostate tumor tissue microarray to determine the levels of CDH7 protein. We have performed knockdown experiments of the *CDH7* mRNA in a prostate cancer cell line. We are in the process of evaluating these prostate cancer cells knocked-down for CDH7 expression for invasive and tumorigenic potential both *in vitro and in vivo*.

15. SUBJECT TERMS
prostate cancer, cadherin, chromosome 18, copy number alteration

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Introduction

Genomic alterations at 18q have been observed in prostate cancer. This research focuses on analyzing the role of copy number increase at 18q22.1 in prostate cancer and the potential function of the critical genes that are found to be present in increased copy numbers. This is innovative research in that we are the first to observe increased copy numbers of genes in this region in prostate cancer. We believe the key genes in increased copy are a class of cell adhesion molecules, the type II mesenchymal cadherins. We are studying the role of overexpression of these genes normally expressed in mesenchymal cells, particularly cadherin 7 (*CDH7*), on the tumorigenic and invasive potential of prostate cancer epithelial cells. These cadherins have never been implicated in prostate cancer, despite the fact that the CDH7 protein is only expressed in brain, testes and prostate. We will analyze the expression of a variety of cadherin gene family members in prostate tumors of varying stages and grades. The results of the study will be able to demonstrate whether overexpression of these mesenchymal-type adhesion molecules is a predisposing factor for prostate cancer development.

Body

The research accomplishments for:

Task 1: Identify the smallest common region of amplification at 18q22.1 in prostate cancer.

a. Perform fluorescence in situ hybridization (FISH) on paraffin-embedded prostate tumor specimens using bacterial artificial chromosomes (BACs) spanning the amplified region.

We previously assembled a complete contig of BAC clones that spanned the original region of altered copy number defined by array comparative genomic hybridization (array CGH). Using FISH methodologies on prostate tumors with hybridization probes created by fluorescently-labeling DNA isolated from two BAC clones that flank the *CDH7* gene, we have shown that the smallest region of altered copy number is a 680 kb region, a portion of which is homologous to the chromosome 18 genome found in the BAC clone RPCI11-775G23, which encodes *CDH7* (Figure 1).

a. **b.**

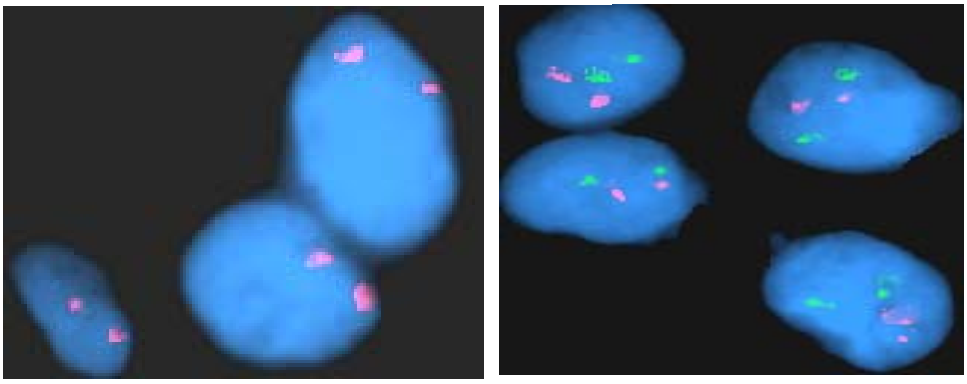

Figure 1: Identification of minimal region of amplification at 18q22.1. A probe proximal to RPCI11-775G23, RPCI11-453M23 (Spectrum Orange) is present in two copies in both tumor (a) and normal prostate tissues (b), while a distal probe RPCI11-425M2 (FITC) is deleted in the prostate tumor section (a). These two probes are present approximately 680 kb apart from each other on the chromosome.

b. Perform quantitative PCR on genomic DNA isolated from prostate tumor specimens.

Since the *CDH7* gene is located within this region of increased copy number, we analyzed the copy number of *CDH7* using quantitative PCR. DNA was isolated from microdissected prostate tumors showing increased copy number at 18q22.1 and a quantitative assay to measure *CDH7* gene copy number using real-time PCR was developed. The copy number of the *CDH7* gene in the prostate tumors ranged between two and seven (data not shown). For the majority of samples, the gene copy number of *CDH7,* as detected by quantitative PCR, correlated with the degree of amplification of the region homologous to RP11-775G23 detected by array CGH in the corresponding tumor section.

c. Perform quantitative reverse transcription-PCR on RNA isolated from prostate tumors to verify increased gene expression with increased gene copy number.

We tested RNA extracted from microdissected prostate tumors to verify whether the genomic amplification of the gene has any impact at the transcriptional level of *CDH7*. As expected, we detected three- to eight-fold overexpression of *CDH7* mRNA in prostate tumors compared to the normal adjacent tissue (Figure. 2). The cell line PZ-HPV-7, derived from prostate epithelial cells, showed very low *CDH7* expression. The expression of *CDH7* was several hundred-fold higher in the tumors, compared to PZ-HPV-7 (Fig. 2). Together, array CGH, FISH and real-time quantitative PCR show increased copy number of the *CDH7* gene in prostate cancer which correlates with increased expression of *CDH7*.

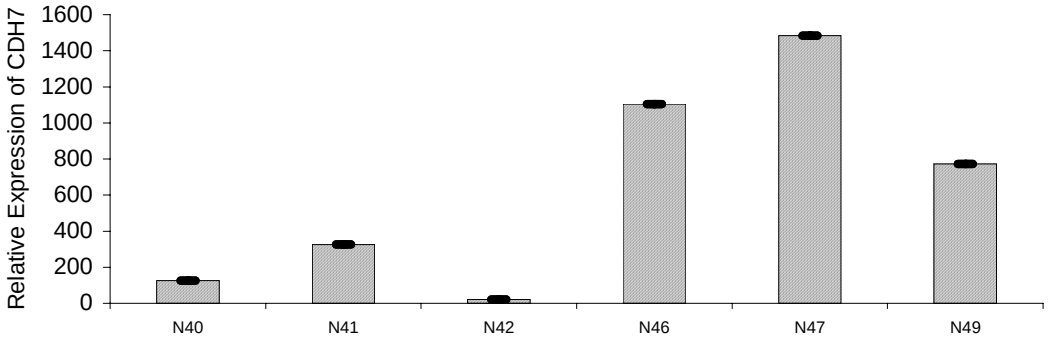

Figure 2: Quantitative real-time RT-PCR analysis of *CDH7* mRNA in prostate tumors. The prostate epithelial cell line PZ-HPV-7 was given a value of 1 for *CDH7* expression and the prostate tumors were shown as fold expression above the level of PZ-HPV-1

Task 1 is completed.

Task 2: Investigate the expression of E-cadherin, N-cadherin, cadherin-7, cadherin-11, cadherin-19 and cadherin-20 proteins in prostate tumors of varying stages and grades.

We are investigating the expression of E-cadherin, N-cadherin and cadherin-11 in prostate tumors because E-cadherin has been found to be down-regulated in prostate tumors (Rubin et al., 2001) and co-expression of two mesenchymal cadherins N-cadherin and cadherin-11 have been reported in prostate cancer samples (Tomita et al., 2000). *CDH7* is within our minimal

region of increased copy number and the cadherin-19 gene is approximately 700 kb distal to the *CDH7.* The gene for another mesenchymal cadherin, cadherin-20 is located 5 megabases proximal to *CDH7* and does not appear to be in increased copy number, but its expression in prostate cancer needs to be assessed. This study will give a more complete picture of changes in cadherin expression during prostate cancer progression.

a. Create antibodies to specifically recognize the type II cadherins (cadherin-7 and -19).

As reported in our first annual report (November 2006), we contracted with Sigma-Genosys to create two custom polyclonal antibodies against CDH7 and CDH19 using unique peptides from the extracellular domain and at the COOH-terminus. We received the antisera and performed some preliminary characterization of the antibodies. Our initial results showed that the antibodies directed against the extracellular domain peptides appeared to recognize the appropriate size of protein from cell lysates, as detected by western blotting. However, prior to their use in immunohistochemistry experiments, the antisera needed further purification. We attempted many types of purification strategies, and a collaborator had success with the antibodies on paraffin-embedded brain tissue, but the antibodies directed against the small peptides were inadequate for immunohistochemical analysis of paraffin-embedded prostate tissue.

Since the peptide antibodies we previously created were not suitable for our purposes, we created mouse monoclonal antibodies directed against bacterially-expressed full-length CDH7 and CDH19 with the help of the UTHSCSA Institutional Antibody core laboratory. We have just completed our initial assessment of purified hybridoma supernatants containing the monoclonal antibodies against human CDH7 and CDH19. Table 1 summarizes our screening results.

Table 1: Summary of anti-CDH7 and anti-CDH19 hybridoma supernatants acceptable for use for western blotting[a] and immunohistochemistry (IHC)[b] experiments.

Anti-CDH7 monoclonal antibodies	Western Blot	IHC	Anti-CDH19 Monoclonal antibodies	Western Blot	IHC
8E2	No	No	4D1	No	No
17G1	Yes	Yes	7D6	No	No
23E5	No	No	16B7	No	No
21F2	No	No	19A8	No	No
25C8	No	No	20BII	No	No
IF8	Yes	Yes	18H7	Yes	No
IID9	No	No	22GII	No	No
IIG8	No	No	5HIIA	Yes	No
8H6	No	No	20CIIB	No	Yes
22GII	No	No	15C1A	No	No
9A9	No	Yes	11D9	No	Yes
162	No	No	1DE6	Yes	No
4D3	No	No	15C1B	No	No
5DI	No	Yes	5HIIB	No	No
5D9	No	No	20CIIA	Yes	Yes
-----	-----		23E5	No	No

[a]Refers to bacterially-expressed fusion protein lysate used for western blot

[b] Refers to 22RV1 prostate cells used for IHC

Figure 3 demonstrates the results we obtained from one immunohistochemistry experiment performed using the 22Rv1 human prostate cancer cell line that contains increased copy number of the chromosome 18 region containing the *CDH7* gene and the anti-CDH7 monoclonal antibody 9A9 as the primary antibody. We are still currently determining the specificity of the antibodies in detecting only CDH7 or CDH19 and not any of the other highly-related cadherin family members. In addition, two commercial suppliers of antibodies (Santa Cruz Biotechnology and AbCam) have developed rabbit polyclonal antibodies against CDH7 protein and we will also be testing these reagents.

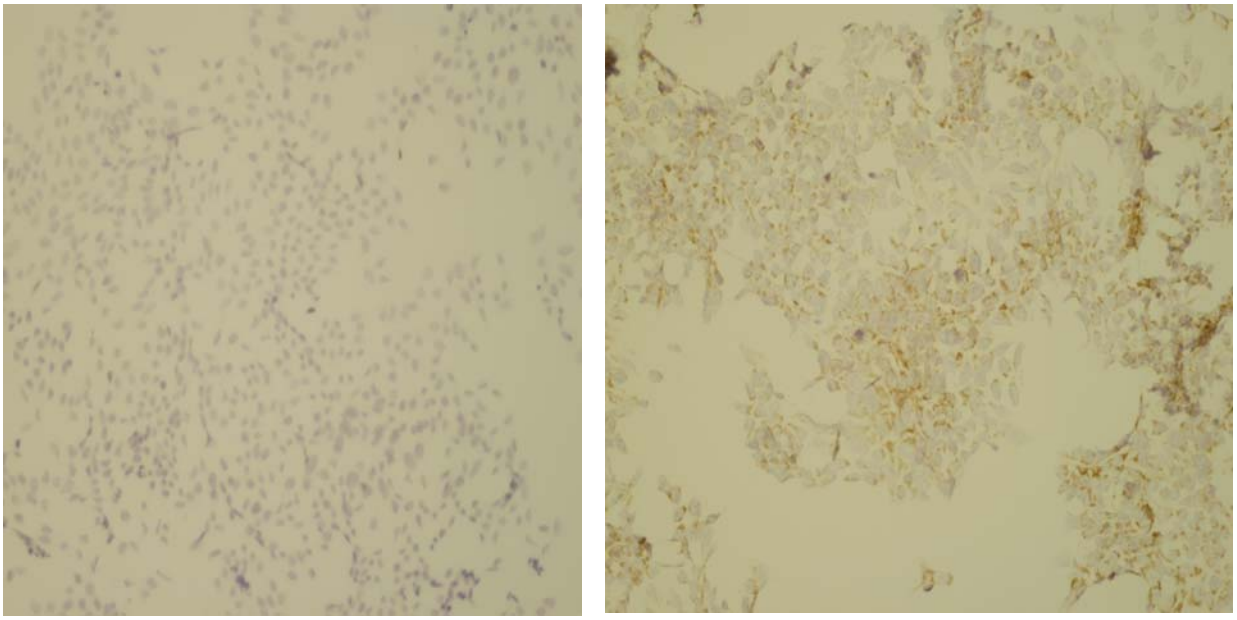

Figure 3: IHC performed using 22RV1 human prostate cancer cells. Left panel, negative control. Right panel, 22RV1 cells incubated with a 1:100 dilution of the anti-CDH7 9A9 hybridoma supernatant

b. Prepare tissue microarrays using prostate cancer specimens of various stages and grades.

Imgenex (San Diego, CA) is a commercial supplier of tissue microarrays and they have developed a prostate tumor tissue microarray consisting of 40 tumors or various stages and Gleason grades with matched normal tissue. FISH was performed on this microarray using a hybridization probe derived from the BAC clone RPCI11-775G23 that contains the *CDH7* gene. All 40 prostate tumors showed three or more signals from the RPCI11-775G23 probe (data not shown). The control probe, a centromeric probe from chromosome 18, showed one to two signals in all 40 tumors on the array indicating that chromosome 18 was not completely amplified in the tumors. These results were consistent with our original array CGH data which showed no correlation between *CDH7* copy number and Gleason score (Table 2). This leads us to hypothesize that the mechanism that results in increased copies of *CDH7* is an early event during tumorigenesis and may be a predisposing factor to the development of prostate cancer.

Table 2. Prostate tumor samples with various pathological grades showing amplification at chromosome 18q22.1

Tumor	Ag	Gleason	Stage	Amplification at 18q22.1
N10	66	6	T2CNXMX	Medium
N12	64	6	T2CNXMX	Mediu
N15	72	7	T2CNXMX	Mediu
N22	59	7	T2CN0M	High
N26	56	6	T2CNXMX	Lo
N30	73	5	T2CNXMX	Mediu
N31	72	6	T2CNXMX	Mediu
N32	54	8	T2CN0M	Lo
N34	52	7	T3BN0M	Norma
N35	56	7	T2CNXMX	Lo
N36	62	6	T2CNXMX	Mediu
N37	62	9	T3AN0M	High
N38	59	6	T2CNXMX	Mediu
N39	60	7	T3NXMX	Lo
N40	70	7	T2CNXMX	High
N41	67	7	T3BN0M	High
N42	67	8	T2CN0M	Lo
N43	65	5	T2BNXMX	Lo
N44	53	7	T2BNXMX	Mediu
N45	55	9	T2BN0M	Lo
N47	71	9	T3BN0M	Lo
N49	56	9	T2CNXMX	Lo

* $_2$ ratio 1-1.5: Low; Log $_2$ ratio 1.5-2.0: Medium; Log $_2$ ratio >2.0: High

c. Perform immunohistochemistry experiments to analyze expression of E-cadherin, N-cadherin and the type II cadherins, cadherin-7, -11, -19 and -20 using tissue microarrays.

Since we had difficulty with our development of antibodies against CDH7 and CDH19 that would be adequate for immunohistochemistry experiments, we were not able to proceed with this task. The preliminary results described under Task 2a lead us to believe that we now have adequate reagents with which to finish this task that includes the analysis of paraffin-embedded prostate tumor tissue sections for CDH7 protein levels. Antibodies to E-cadherin, N-cadherin and cadherin-11 are commercially-available and the amount of protein of these cadherins will also be analyzed to provide a more complete picture of the cadherin expression changes occurring during the tumorigenic process in prostate.

d. Perform immunohistochemistry experiments to test the tissue specificity of increased copy number of the three type II cadherins (cadherin-7, -19 and –20).

We performed FISH experiments with the RPCI11-775G23 (contains *CDH7*) probe on tissue microarrays consisting of cancer from 12 organ sites (Imgenex common cancers A and B) The gene copy number alteration detected on chromosome 18q22.1 using probe RPCI11-775G23 on prostate tumor samples was not observed in any other tumors samples, including stomach, esophagus, lung, colon, thyroid, kidney, breast, liver, urinary bladder, ovary and pancreas.

These data indicate that the increased copy number of the RPCI11-775G23 region containing *CDH7* is tumor specific, and is limited to the prostate. Of these 12 tissues analyzed, only prostate is known to express *CDH7* (Kools et al., 2000). Since *CDH7* is also expressed in testes (Kools et al., 2000), we have analyzed a small number of testicular tumors by FISH and have shown that *CDH7* is also in increased copy number.

Tasks 2a, b and d are completed.

Task 3: Knockdown expression of type II cadherins in prostate cancer cell lines and analyze the phenotype of the cells for invasive and tumorigenic potential.

a. Create transient and stable RNA interference constructs and perform experiments to knockdown expression of cadherin-7, -19 or -20, individually.

We designed short hairpin RNAs (shRNA) for cadherin-7, -19 and -20 using design tools provided by Ambion (The Woodlands, TX). The shRNA was cloned into the pSilencer vector (Ambion) which contains a mammalian selectable marker for creating RNA interference constructs that can be stably selected in a mammalian cell line. The shRNA construct for *CDH7* was stably transfected into the 22Rv1 human prostate cancer cell line, a cell line that expresses *CDH7*. Analysis of the *CDH7* mRNA in the stable transfectants showed dramatic reduction in the mRNA levels, but these results need to be confirmed by performing western blots using our newly-developed monoclonal antibodies to determine the levels of CDH7 protein.

b. Analyze the transformed metastatic phenotype of the prostate cancer cells after knockdown of the type II cadherins using *in vitro* assays for anchorage independence and migration/invasion.

We have evaluated two *in vitro* assays from Calbiochem (Innocyte; Gibbstown, NJ) for determining the migratory and invasive potential of the parental 22Rv1 prostate cancer cell line. The Innocyte migration assay is an assay for cell migration in 24-well plates. Cells migrate through an 8 μm pore to a feeder layer containing serum as a chemoattractant. Migrated cells are quantitated using a cell-permeable fluorescent dye. The Innocyte invasion assay quantitatively measures cell invasion. The assay contains cell culture inserts with an 8 μm polycarbonate membrane. This membrane is coated with a thin layer of a biological matrix. This layer prevents noninvasive cells from going through the membrane. Cells are quantified utilizing a highly sensitive fluorescent dye. Preliminary results from the migration assay using 22Rv1 cells demonstrated that there was a 10-fold increase in cell migration when media containing 10% serum was used as a chemoattractant compared to serum free media (data not shown). In the invasion assay, 22Rv1 cells did not show invasive potential using serum as a chemoattractant (data not shown). We are currently testing the 22Rv1 cells knocked-down for expression of CDH7 in the migration and invasion assays.

c. Analyze the *in vivo* tumorigenic phenotype of the prostate cancer cells after knockdown of type II cadherin mRNA.

Due to our problems in creating antibodies that demonstrated successful knockdown of CDH7 mRNA, no work to date has been accomplished on Tasks 3b or 3c. Now that we have adequate antibodies showing that the cells do not express CDH7 protein, we are ready to start

these experiments. We have experience in performing the in vivo analysis of tumorigenic phenotype, so these experiments will be completed quickly.

Task 3a is completed.

Due to the difficulties we encountered with the antibody production, we requested and were approved for a one year no cost extension. This time will allow us to finish Tasks 2 and 3 as originally proposed.

Key Research Accomplishments (To Date)

- **Defined a minimal region of increased copy number (680kb) of 18q22.1 containing the cadherin 7 gene in prostate cancer.**
- **Determined that increased copy number of the cadherin-7 gene results in increased cadherin-7 mRNA levels in prostate tumors**
- **Determined that increased copy number of the cadherin-7 gene does not correlate with the stage or Gleason score of prostate tumors.**
- **Determined that increased copy number of the cadherin-7 gene is found specifically in prostate cancer and not 11 other common cancers.**
- **Created and mouse monoclonal antibodies to full-length cadherin-7 and -19 proteins.**
- **Knocked down expression of the cadherin-7 gene in the prostate cancer cell line 22Rv1 that contains increase copy number of the cadherin-7 gene.**
- **Evaluated *in vitro* assays and screened 22Rv1 cells for migratory and invasive potential.**

Reportable Outcomes

We previously submitted our work as a manuscript to the scientific journals Genes, Chromosomes and Cancer and Oncogene, however without data demonstrating the amount of CDH7 protein in the prostate tumors our manuscript has not been judged acceptable for publication (included in the appendix in first annual report – November 2006). With our new monoclonal antibodies derived in Task 2a, we will complete the manuscript and make it acceptable for publication. Our results have been presented as a poster at the DOD Innovative Minds in Prostate Cancer Today meeting held in Atlanta, GA in September 2007 (included in the appendix in the second annual report – November 2007).

Conclusions

We demonstrate that the region of increased copy number in prostate cancer at 18q22.1, originally detected by array CGH, can be minimized to a 680 Kb region that contains the cadherin-7 gene. This increased copy number of the cadherin-7 gene is specific to prostate cancer and is not found in 11 other common cancers. The increased copy number of the cadherin-7 gene also results in increased levels of cadherin-7 mRNA in prostate tumors. We have created monoclonal antibodies against cadherin-7 to use in immunohistochemistry experiments to determine if the increase in gene copies affects the level of protein. We have performed knockdown experiments of the cadherin-7 mRNA in a prostate cancer cell line and we will be analyzing the cadherin-7 protein levels in these cells. We will use *in vitro* assays to evaluate whether the cells lines with reduced cadherin-7 protein expression exhibit altered invasive or tumorigenic potential.

We have not found that increased number of the cadherin-7 gene is correlated with the stage or Gleason score of the prostate tumors and may be an early marker of prostate cancer. Since the cadherins are a class of cell adhesion molecules and the type II cadherins are mesenchymal cadherins not normally expressed in epithelial cells, the increased expression of cadherin-7 may be a marker of the tumorigenic phenotype. Assays to detect cadherin-7 protein in prostate tissue have the potential to be developed into clinically-useful biomarkers for prostate cancer.

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